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# Effects of Atrazine on Estrogen Receptor $\alpha$ - and G Protein-Coupled Receptor 30-Mediated Signaling and Proliferation in Cancer Cells and Cancer-Associated Fibroblasts

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**Editor's Note:** In the February 2014 issue of *Environmental Health Perspectives*, Albanito et al. retracted their article "G-Protein-Coupled Receptor 30 and Estrogen Receptor- $\alpha$  Are Involved in the Proliferative Effects Induced by Atrazine in Ovarian Cancer Cells" because they discovered that errors were introduced during the sorting of files related to the presentation of the Western blots in Figures 6 and 8 [Environ Health Perspect 122:A42 (2014); doi:10.1289/ehp.11297RET]. There was no evidence that the errors that led to the retraction were intentional, and the authors were forthcoming in acknowledging the errors. Thus, *EHP* agreed to consider a new manuscript on this topic. The paper "Effects of Atrazine on Estrogen Receptor  $\alpha$ - and G Protein-Coupled Receptor 30-Mediated Signaling and Proliferation in Cancer Cells and Cancer-Associated

Fibroblasts” [Environ Health Perspect (2015); doi:10.1289/ehp.1408586] contains corrected Western blots as well as new data. The manuscript was handled as a new submission and underwent *EHP*'s standard peer-review process.

**Running title:** Atrazine action in ovarian cancer cells and CAFs.

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## **Abstract**

**Background:** The pesticide atrazine does not bind or activate the classical estrogen receptor (ER), but up-regulates the aromatase activity in estrogen-sensitive tumor cells. It has recently been reported that the G protein estrogen receptor (GPR30/GPER) is involved in certain biological responses to endogenous estrogens and environmental compounds exerting estrogen-like activity.

**Objectives:** We aimed to evaluate the potential of atrazine to trigger the GPER-mediated signaling in cancer cells and cancer-associated fibroblasts (CAFs).

**Methods and Results:** Using gene reporter assays in diverse types of cancer cell, we found that atrazine does not transactivate endogenous ER $\alpha$  or chimeric proteins that encode the ER $\alpha$  and ER $\beta$  hormone binding domains. Conversely, atrazine was able to bind to GPER to induce ERK activation and the expression of estrogen target genes, which interestingly relied on both GPER and ER $\alpha$  expression. As a biological counterpart, atrazine stimulated the proliferation of ovarian cancer cells depending on GPER and ER $\alpha$ , as evidenced by gene silencing experiments and using specific signaling inhibitors. Of note, atrazine through GPER elicited ERK phosphorylation, gene expression and migration in CAFs, thus extending its stimulatory role to these main players of the tumor microenvironment.

**Conclusions:** The current results suggest a novel mechanism through which atrazine may exert relevant biological effects in cancer cells and CAFs. On the basis of our data, atrazine should be included among the environmental contaminants that may elicit estrogenic activity through the GPER-mediated signaling.

## Introduction

Atrazine belongs to the 2-chloro-*s*-triazine family of herbicides and is one of the most common pesticide contaminants of groundwater and surface water (Miller et al. 2000). People who work in agriculture or reside near agricultural fields may have higher levels of exposure to atrazine through spray drift than the general population (Gammon et al. 2005). Occupational exposure to atrazine may occur during manufacturing, formulation operations and application, whereas non-occupational exposure might arise from drinking water or diet. In particular, the absorbed dosage values ranged from 1.8 to 6.1  $\mu\text{g}/\text{kg}/\text{day}$  (Gammon et al. 2005). Moreover, atrazine is not removed from the body within 24hr, as its metabolites are still detected in urine 48hr after an oral dose (Davidson 1988). Therefore, pathophysiological effects may occur after repeated dosing and result from an accumulation above a critical threshold.

Epidemiologic studies have associated long-term exposure to triazine herbicides with increased risk of ovarian cancer in female farm workers in Italy (Donna et al. 1989) and breast cancer in the general population of Kentucky in the United States (Kettles et al. 1997). In addition, atrazine leads to tumor development in the mammary gland and reproductive organs of female F344 rats (Pintér et al. 1990). Given the potential ability of atrazine to interfere with reproduction and to cause cancer, the European Union banned its use (Sass and Colangelo 2006). However, the U.S. Environmental Protection Agency has approved the use of atrazine considering the lack of a clear association between the levels of exposure and cancer incidence in pesticide applicators (Sass and Colangelo 2006).

Previous studies have demonstrated that triazine herbicides are not able to bind to or activate the classical estrogen receptor (ER) (Connor et al. 1996; Tennant et al. 1994). In recent years, increasing evidence has demonstrated that steroid hormones, including estrogens, can exert rapid

actions interacting with receptors located within or near the cell membrane (Norman et al. 2004). Moreover, it has been suggested that nongenomic estrogen actions, like genomic ones, may be triggered by environmental estrogens (Thomas 2000). Of note, these compounds compete with [3H]E2 for binding to estrogen membrane receptors and exert agonist effects through diverse transduction pathways in different cell contexts (Nadal et al. 2000). In addition, a seven-transmembrane receptor, namely GPR30/GPER, has been shown to mediate relevant biological responses to estrogens (Maggiolini and Picard 2010). In this regard, our and other studies have demonstrated that GPER is involved in multiple actions triggered by estrogenic compounds, including environmental contaminants, in a variety of cancer cells as well as in cancer-associated fibroblasts (CAFs) (Albanito et al. 2007; Lappano et al. 2010; Madeo and Maggiolini 2010; Pandey et al. 2009; Pupo et al. 2012; Vivacqua et al. 2006a; Vivacqua et al. 2006b).

In the present study, we demonstrate that gene expression changes and growth effects induced by atrazine in ovarian cancer cells rely on both GPER and ER $\alpha$ . Furthermore, we show that GPER alone is able to mediate the stimulatory effects exerted by atrazine in ER-negative SkBr3 breast cancer cells and CAFs.

## **Methods**

### **Reagents**

Atrazine, 2-chloro-4-(ethylamine)-6-(isopropylamine)-s-triazine, 17 $\beta$ -estradiol (E2), N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89), Wortmannin (WM), and PD98059 (PD) were purchased from Sigma-Aldrich (Milan, Italy); AG1478 (AG) and 1-tert-Butyl-3-(4-chlorophenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP2) from Biomol Reaserch Laboratories (DBA, Milan, Italy), ICI 182,780 (ICI) from Tocris Chemicals (Bristol,

United Kingdom) and GF109203X (GFX) from Calbiochem (VWR International, Milan, Italy). All compounds were solubilized in DMSO, except E2 and PD, which were dissolved in ethanol.

### **Cell culture**

Human BG-1 and 2008 ovarian cancer cells (kindly provided by D. Picard, University of Geneva, Switzerland) as well as human Ishikawa endometrial cancer cells (kindly provided by D. Picard, University of Geneva, Switzerland) were maintained in Dulbecco's Modified Eagle Medium (DMEM) without phenol red supplemented with 10% fetal bovine serum (FBS) and 100µg/ml penicillin/streptomycin. Human MCF-7 and SkBr3 breast cancer cells were maintained in DMEM with phenol red and RPMI 1640 without phenol red, respectively, supplemented with 10% FBS and antibiotics. CAFs were extracted as previously described (Madeo and Maggiolini 2010). Briefly, breast cancer specimens were collected from primary tumors of patients who had undergone surgery. Signed informed consent was obtained from all the patients and from the institutional review board(s) of the Regional Hospital of Cosenza. Tissues from tumors were cut into smaller pieces (1-2 mm diameter), placed in digestion solution (400 IU collagenase, 100 IU hyaluronidase, and 10% serum, containing antibiotic and antimycotic solution), and incubated overnight at 37°C. Cells were then separated by differential centrifugation at 90×g for 2min. Supernatant containing fibroblasts was centrifuged at 485×g for 8min; the pellet obtained was suspended in fibroblasts growth medium (Medium 199 and Ham's F12 mixed 1:1, supplemented with 10% FBS and antibiotics) and cultured at 37°C in 5% CO<sub>2</sub>. Primary cells cultures of breast fibroblasts were characterized by immunofluorescence (data not shown) as described previously (Pupo et al. 2012).

Cells were switched to medium without serum the day before immunoblots and reverse transcription-PCR (RT-PCR) experiments.

### **Plasmids and luciferase assays**

Firefly luciferase reporter plasmids used were ERE-luc for ER $\alpha$  (Bunone et al. 1996) and GK1 for the Gal4 fusion proteins (Gal-ER $\alpha$  and Gal-ER $\beta$ ) (Webb et al. 1998; Seipel et al. 1992). The Renilla luciferase expression vector pRL-TK (Promega, Milan, Italy) was used as a transfection standard. On the day before transfection, cells ( $1 \times 10^5$ ) were plated into 24-well dishes in regular medium, which was replaced with medium supplemented with 1% charcoal-stripped (CS) FBS lacking phenol red on the day of transfection. Transfections were performed using the XtremeGene 9 reagent as recommended by the manufacturer (Roche Diagnostics, Germany) with a mixture containing 0.3 $\mu$ g of reporter plasmid, 1ng of pRL-TK and 0.1 $\mu$ g of effector plasmid where applicable. After 5-6 hr, ligands were added and cells were incubated for 16-18 hr. Luciferase activity was measured with the Dual Luciferase kit (Promega Italia, Milan, Italy) according to the manufacturer's recommendations. Firefly luciferase values were normalized to the internal transfection control provided by the Renilla luciferase activity. Normalized relative light unit values obtained from cells treated with vehicle were set as 1-fold induction upon which the activity induced by treatments was calculated.

### **Gene silencing experiments**

Cells were plated onto 10cm dishes and transfected for 24hr before treatments using XtremeGene 9 reagent (Roche Diagnostics, Milan, Italy). The ER $\alpha$  siRNA and the respective control were purchased from Sigma-Aldrich. The short hairpin (sh)RNA constructs to knock down the expression of GPER and CTGF and the unrelated shRNA control constructs have been described previously (Pandey et al. 2009).

## **RT-PCR**

The evaluation of gene expression was done by semiquantitative RT-PCR, as previously described (Maggiolini et al. 1999). Briefly, quantitative RT-PCR involves direct incorporation of digoxigenin-11-dUTP (DIG-dUTP) during amplification of cDNAs, separation of RT-PCR products by agarose gel electrophoresis, Southern transfer to a nylon membrane, and chemiluminescent detection with an anti-DIG antibody. For the sequences of primer used see Supplemental Material, Table S1.

## **Western blotting**

Cells were grown in 10-cm dishes, exposed to ligands, and then lysed as previously described (Pandey et al. 2009). Protein concentrations were determined using Bradford reagent (Sigma-Aldrich) according to the manufacturer's recommendations. Equal amounts of whole protein extract were resolved on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences). Membranes were probed overnight at 4°C with antibodies against ER $\alpha$  (F-10, sc-8002), GPER (N-15, sc-48525-R), c-fos (H-125, sc-7202), CTGF (L-20, sc-14939),  $\beta$ -actin (AC-15, sc-69879), phosphorylated ERK1/2 (E-4, sc-7383) and ERK2 (C-14, sc-154) (Santa Cruz Biotechnology), and then revealed using the ECL System (GE Healthcare, Milan, Italy).

## **c-Src kinase assay**

Cell lysates were incubated with ~1  $\mu$ g/ml mouse monoclonal anti-c-Src antibody (clone 327, ab16885, Abcam) overnight at 4°C, then added to an equal amount of goat anti-mouse IgG antibodies and incubated for additional 30 min. Then, 40  $\mu$ l of a 50% suspension of protein G-Sepharose were added and incubation continued for an additional 30 min. The samples were centrifuged and pellets washed with 1 ml of lysis buffer four times and used for c-Src kinase

assays. The activity of c-Src kinase was assayed using acidified enolase (0.5 mg/ml) as a substrate (Di Domenico et al. 1996).

### **Ligand binding assay**

SkBr3 cells were grown in 10-cm cell culture dishes and incubated with 50nM [2,4,6,7-<sup>3</sup>H]E2 (89 Ci/mmol; GE Healthcare, Milan, Italy) in the presence or absence of increasing concentration of nonlabeled E2 or atrazine for 2hr at 37°C. Then, cells were washed with ice-cold PBS and the radioactivity collected by 100% ethanol extraction was measured by liquid scintillation counting. The displacement of [<sup>3</sup>H]E2 binding by the competitors was expressed as a percentage of the maximum specific binding of E2.

### **Proliferation assay**

Cells were seeded in 24-well plates in regular growth medium. After cells attached, they were incubated in medium containing 2.5% charcoal-stripped FBS, transfected for 24hr and treated as indicated, renewing transfection and treatments every 2 days. Cells were counted using an automated cell counter (Life-Technologies) following the manufacturer's recommendations.

### **Cell cycle analysis**

Cells synchronized for 24h in serum-free medium were transfected, treated for 8hr with treatments and subjected to fluorescence-activated cell sorting (FACS) analysis. Adherent and floating cells were centrifuged and resuspended in PBS containing 20µg/ml propidium iodide plus 40µg/ml ribonuclease (Sigma-Aldrich) for 1h. Cells were then subjected to FACS analysis (FACS Jazz, BD, Milan, Italy) and results were expressed in terms of percentage.

### **Transwell cell migration assay**

Migration assay was performed in CAFs using Boyden chambers (Costar Transwell, 8 mm polycarbonate membrane, Sigma-Aldrich). For knockdown experiments, cells were transfected for 24hr, then seeded in the upper chambers. Treatments were added to the serum-free medium in the bottom wells. After 24hr, cells on the bottom side of the membrane were fixed, stained with GIEMSA (Sigma-Aldrich), photographed and counted.

### **Statistical analysis**

Statistical analysis was done using ANOVA followed by Newman-Keuls' testing to determine differences in means.  $P < 0.05$  was considered as statistically significant.

## **Results**

### **ER $\alpha$ and ER $\beta$ activation**

Based on the evidence that atrazine influences the development of estrogen-sensitive tumors (Cooper et al. 2007), we first evaluated whether atrazine could activate a transiently transfected ER reporter gene in estrogen-sensitive ovarian BG-1, breast MCF-7 and endometrial Ishikawa cancer cells. E2 treatment induced a strong ER $\alpha$  transactivation, which was prevented using the ER antagonist ICI (Figure 1A-C). On the contrary, atrazine failed to stimulate the luciferase expression or to block the activation of ER $\alpha$  by E2 (Figure 1A-C). Likewise, an expression vector encoding ER $\alpha$  transfected in ER-negative SkBr3 breast cancer cells was not activated by atrazine (Figure 1D). In order to confirm that atrazine does not act as an ER $\alpha$  agonist and to examine whether ER $\beta$  could respond to atrazine, we turned to a heterologous system. Chimeric proteins consisting of the DNA binding domain of the yeast transcription factor Gal4 and the ER $\alpha$  or ER $\beta$  hormone binding domain, which were transiently transfected in SkBr3 cells,

showed a strong transactivation by E2 but not by atrazine (Figure 1E,F) confirming that atrazine does not transactivate ER.

### **GPER binding and ERK phosphorylation**

Considering that diverse environmental contaminants exhibit binding affinity for GPER (Thomas and Dong 2006), we performed ligand binding studies using radiolabeled E2 as a tracer in ER-negative and GPER-positive SkBr3 breast cancer cells (Lappano et al. 2010; Lappano et al. 2012a; Lappano et al. 2012b). Of note, atrazine displaced the tritiated E2 in a dose-dependent manner (Figure 2A), demonstrating the ability to bind to GPER, even though with a lower binding affinity respect to E2.

Numerous reports have recently demonstrated that estrogens and xenoestrogens can generate rapid signalling via second messengers such as  $\text{Ca}^{2+}$ , cAMP, nitric oxide and G-proteins, which in turn activate numerous downstream kinases (Bulayeva and Watson 2004; Lappano and Maggiolini 2011). In this regard, we found that atrazine stimulates ERK phosphorylation in BG-1 cells like E2 (Figure 2B,C). Hence, we performed time-course experiments using specific pharmacological inhibitors in BG-1 and 2008 ovarian cancer cells exhibiting a similar receptor expression pattern (Safei et al. 2005). As shown in Figure 3 (A,D), E2 and atrazine induced ERK phosphorylation in a time-dependent manner in both ovarian cancer cell lines. The treatment with the ER antagonist ICI, the EGFR inhibitor AG and the ERK inhibitor PD prevented ERK activation upon exposure to E2 and atrazine, whereas GFX, H89 and WM, inhibitors of protein kinase C (PKC), protein kinase A (PKA) and phosphoinositide 3-kinase (PI3K), respectively, did not (Figure 3B,C,E,F). Taken together, the inhibitory effects elicited by ICI, AG and PD suggest that the EGFR/ERK transduction pathway and  $\text{ER}\alpha$  are involved in ERK activation induced by E2 and atrazine.

### **mRNA expression of estrogen target genes**

Next, we evaluated in BG-1 cells the ability of atrazine to regulate the expression of genes that have been shown to respond to estrogens and environmental contaminants (Pandey et al. 2009; Pupo et al. 2012). To this end, we performed semiquantitative RT-PCR experiments comparing the mRNA levels after standardization with a housekeeping gene encoding the ribosomal protein 36B4. Upon 1hr treatment, atrazine enhanced *c-fos*, *CTGF* and *cyclin A* levels although to a lesser extent than E2. E2 also stimulated the expression of *PR*, *pS2* and *cyclin D1* (see Supplemental Material, Table S2). A 24hr treatment with atrazine increased *PR*, *pS2* and *cyclin A* levels, whereas E2 induced not only the expression of these genes but also the expression of *c-fos*, *cathepsin D*, *cyclin D1* and *cyclin E* (see Supplemental Material, Table S2). Similar results were obtained also in 2008 cells (data not shown). It has been shown that estrogens may signal through intracellular effectors, like c-Src, which in turn activate complex transduction networks leading to gene expression changes in cancer cells (Migliaccio et al 2007). Hence, we used the c-Src inhibitor namely PP2 in order to evaluate the involvement of c-Src in gene transcription stimulated by atrazine and E2 in BG-1 (see Supplemental Material, Table S3) and 2008 cells (data not shown). The up-regulation of *PR*, *pS2* and *Cyclin A* induced by E2 was lowered in the presence of PP2, although the induction of these genes still remained statistically significant. On the contrary, the gene transcription prompted by atrazine was not influenced using PP2. In accordance with these findings, atrazine did not enhance c-Src kinase activity on enolase as determined in BG-1 cell lysates immunoprecipitated with anti-c-Src antibody (data not shown).

### **Transduction pathways involved in the up-regulation of c-fos protein levels**

Using c-fos expression as a molecular sensor of atrazine action, we sought to determine whether atrazine could also regulate c-fos at protein level and the transduction pathways involved in this

response. Interestingly, the up-regulation of *c-fos* observed in BG-1 and 2008 cells treated for 2hr with E2 (see Supplemental Material, Figure S1, panels A and C) and atrazine (see Supplemental Material, Figure S1, panels B and D) was abolished using the ER antagonist ICI, the EGFR inhibitor AG and the ERK inhibitor PD (see Supplemental Material, Figure S1). On the contrary, GFX, H89 and WM, inhibitors of PKC, PKA and PI3K, respectively, did not interfere with *c-fos* stimulation (see Supplemental Material, Figure S1). Altogether, these results suggest that in ovarian cancer cells atrazine triggers *c-fos* protein increase through ER $\alpha$  and the EGFR-MAPK transduction pathway, thus confirming the results obtained in the ERK activation studies. On the basis of these and our previous data showing that *c-fos* stimulation by E2 occurs through GPER and ER $\alpha$  in cancer cells expressing both receptors (Albanito et al. 2007), we examined whether atrazine could act in a similar manner. Silencing ER $\alpha$  (see Supplemental Material, Figure S2, panels A, B, E, F) or GPER (see Supplemental Material, Figure S2, panels C, D, G, H) in both BG-1 and 2008 cells, E2 and atrazine did not display the ability to induce the expression of *c-fos* and *CTGF* which is a main GPER-target gene (see Supplemental Material, Figure S2). Next, to evaluate whether atrazine could induce a rapid response in an ER-negative and GPER-positive cell context, we used the SkBr3 breast cancer cells. The ERK phosphorylation and the induction of *c-fos* and *CTGF* observed upon stimulation with atrazine (see Supplemental Material, Figure S3) and E2 (data not shown) were abolished knocking-down the expression of GPER, reminiscing our previous data obtained using estrogens (Maggiolini et al. 2004; Lappano et al. 2010; Lappano et al. 2012a; Lappano et al. 2012b).

### **BG-1 and 2008 cell proliferation and cell cycle analysis**

Then, we determined that E2 and atrazine induce the proliferation of BG-1 and 2008 cells in a concentration-dependent manner (Figure 4A,E). The growth effects elicited by E2 and atrazine

were no longer evident in the presence of AG and PD (Figure 4B,F) and silencing the expression of GPER or ER $\alpha$  (Figure 4C,D,G,H), suggesting that both receptors along with the EGFR/MAPK transduction pathway contribute to the proliferation induced by atrazine. To further corroborate these results, we performed cell cycle analysis showing that the increase of BG-1 cells in G2/M phase induced by atrazine is prevented by silencing the expression of ER $\alpha$  or GPER (see Supplemental Material, Figure S4).

### **ERK phosphorylation, gene expression changes, and migration in CAFs**

In order to provide further evidence regarding the ability of atrazine to trigger biological responses through GPER, we evaluated the activity of this contaminant in CAFs obtained from breast tumor patients (Madeo and Maggiolini 2010). In these cells that express GPER and lack ER $\alpha$  (Figure 5 and see Supplemental Material, Figure S5), E2 and atrazine stimulated through GPER ERK phosphorylation and the expression of both c-fos and CTGF (Figure 5A,B). In addition, the migration of CAFs promoted by E2 and atrazine was abolished knocking-down the expression of GPER or CTGF (Figure 5C) which exerts an acknowledged role in the migration of cancer cells (Pandey et al. 2009). Collectively, these results indicate that atrazine induces relevant biological effects through GPER also in CAFs that mainly contribute to worse cancer features as key players within the tumor microenvironment (Bhowmick et al. 2004).

### **Discussion**

In the present study, we demonstrated for the first time that atrazine exerts an estrogen-like activity in ovarian, breast cancer cells and CAFs through GPER, which mediates estrogen signals as largely reported in previous studies (reviewed in Prossnitz and Barton 2014).

Previous studies have demonstrated that atrazine elicits an estrogen action up-regulating the aromatase activity in certain cancer cells with elevated aromatase levels (Sanderson et al. 2001), but not by binding to or activating ER $\alpha$  (Connor et al. 1996; Roberge et al. 2004; Tennant et al. 1994). Using different model systems, we confirmed that atrazine did not activate ER $\alpha$  although it induces the expression of several estrogen target genes. In this regard, it should be mentioned that a previous study reported the recruitment of ER $\alpha$  by distinct compounds and growth factors to gene promoter sequences different from the classical estrogen responsive elements (Dudek and Picard 2008). Furthermore, our data show that GPER and ER $\alpha$  along with the EGFR/MAPK pathway contribute to the biological responses to atrazine in diverse cancer cells. Thus, the current findings indicate that a complex interplay between the different estrogen receptors and transduction pathways contributes to atrazine activity, which nevertheless may be still evident in cell contexts expressing only GPER, like SkBr3 cells and CAFs.

The current study corroborates our previous data regarding the physical interaction between GPER and ER $\alpha$  and the biological responses triggered by the functional cross-talk of these receptors (Vivacqua et al. 2009). Previous research (Migliaccio et al. 2006; Vivacqua et al. 2009) showed that EGFR co-immunoprecipitates with ER $\alpha$  and GPER, suggesting that an intricate cross-talk may occur among these main transduction mediators in cancer cells. Collectively, these findings indicate that estrogens and estrogen-like compounds may exert pleiotropic actions through ER $\alpha$  in a direct manner as well as via the GPER-EGFR transduction signalling, which may engage ER $\alpha$  towards the stimulation of cancer cells. In accordance with these observations, in BG-1 and 2008 cancer cells the silencing of GPER or ER $\alpha$  expression and the inhibition of the EGFR/ERK signalling prevented the action of atrazine, confirming that a cooperation between these receptors is involved in the biological responses to atrazine. In addition, the present data

are in accordance with previous studies showing that xenoestrogens may mimic estrogen action in several animal and cell models (Bulayeva and Watson 2004; Nadal et al. 2000).

It has been largely reported that a subset of estrogen-sensitive cell tumors can proliferate regardless of ER expression. Under these conditions, which may be represented by SkBr3 breast cancer cells, the GPER/EGFR signalling could allow stimulatory effects by environmental estrogens, as shown in the present and previous investigations (Maggiolini and Picard 2010; Pupo et al. 2012). Thus, multiple transduction pathways triggered at the membrane level as well as within the diverse cell types contribute to the nature and the magnitude of biological responses to distinct estrogenic compounds. Consequently, these agents should be examined towards their complex mechanistic and functional outcomes which result from an interaction with a repertoire of different receptor proteins.

Here, we have provided novel insights regarding the potential role of GPER in mediating the action of atrazine not only in estrogen-sensitive tumors but also in CAFs. As CAFs play a key role within the tumor microenvironment as well as at metastatic sites (Bhowmick et al. 2004), our results further extend the molecular mechanisms through which atrazine may contribute to cancer progression. However, future studies are required in order to evaluate the effects exerted by atrazine *in vivo* through GPER in cancer progression as well as in other pathophysiological conditions, as these investigations are still lacking.

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## Figure Legends

**Figure 1.** ER $\alpha$  transactivation in BG-1 (A), MCF-7 (B), and Ishikawa (C) cells transfected with the ER luciferase reporter plasmid (ERE-luc) and treated with 100nmol/L E2, 1 $\mu$ mol/L atrazine (Atr) and 10 $\mu$ mol/L ER antagonist ICI, as indicated. Luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle (–) were set as 1-fold induction, upon which the activity induced by treatments was calculated. SkBr3 cells were transfected with ERE-luc and ER $\alpha$  expression plasmid (D) or with Gal4 reporter gene (GK1) and the Gal4 fusion proteins encoding the HBD of ER $\alpha$  (GalER $\alpha$ ) (E) and ER $\beta$  (GalER $\beta$ ) (F) and treated with 100nmol/L E2, 1 $\mu$ mol/L Atr and 10 $\mu$ mol/L ICI, as indicated. Values shown are mean  $\pm$  SD of three independent experiments performed in triplicate. ( $\circ$ ) indicates  $p < 0.05$  for cells receiving vehicle (–) versus treatments.

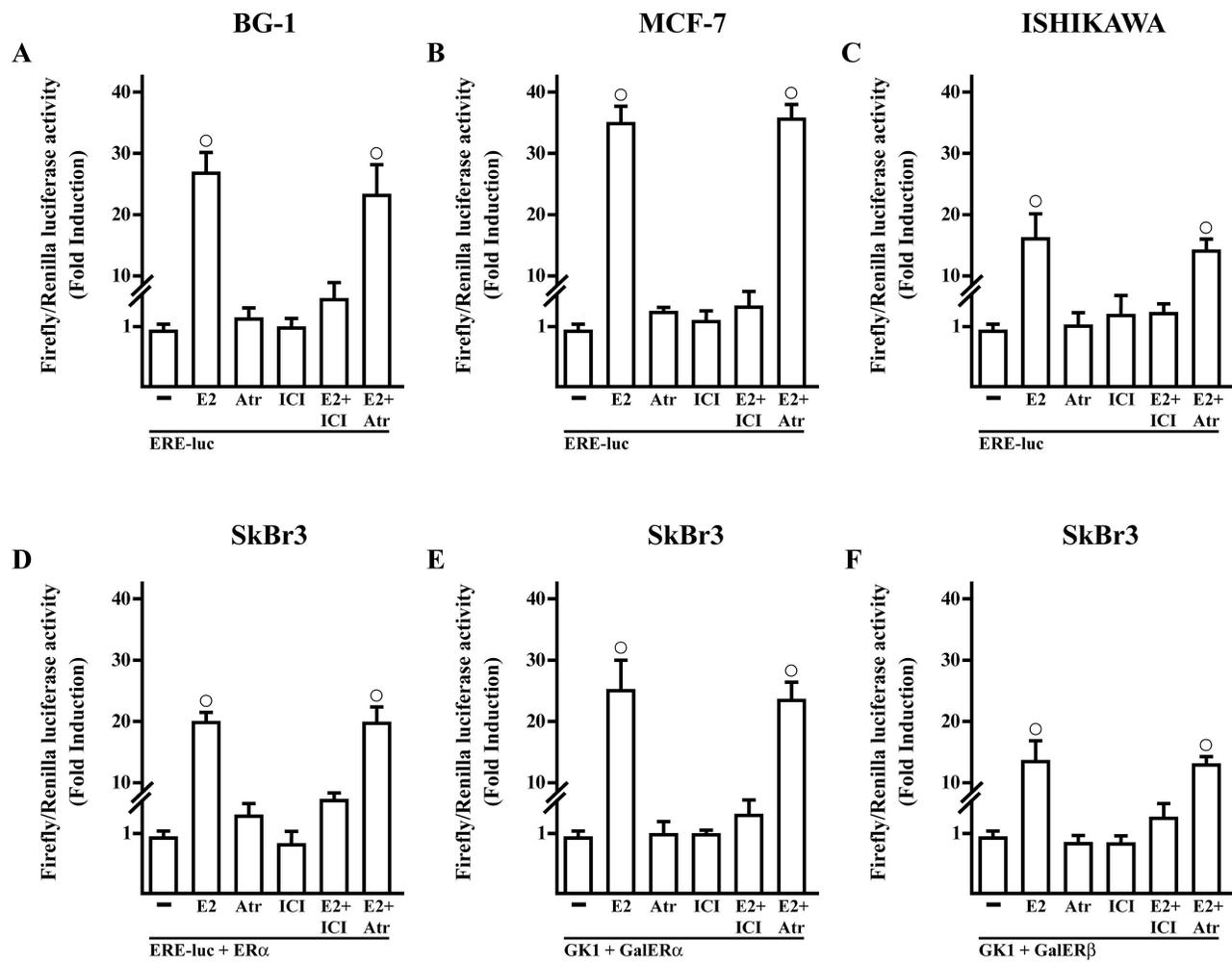
**Figure 2.** (A) Ligand binding assay for GPER in SkBr3 cells. Competition curves of increasing concentration of unlabeled E2 and atrazine (Atr) expressed as a percentage of maximum specific [ $^3$ H]E2 binding. Each data point represents the mean  $\pm$  SD of three separate experiments performed in triplicate. ERK1/2 phosphorylation in BG-1 cells exposed to increasing concentrations of E2 (B) or Atr (C) for 20 min. ERK2 serves as a loading control. Data shown are representative of three independent experiments.

**Figure 3.** ERK1/2 phosphorylation in BG-1 (A) and 2008 (D) cells treated with vehicle (–), 100nmol/L E2 and 1 $\mu$ mol/L atrazine (Atr) for the indicated times. ERK1/2 phosphorylation in BG-1 (B,C) and 2008 (E,F) cells treated for 20 min as indicated with vehicle (–), 100nmol/L E2, 1 $\mu$ mol/L Atr, alone and in combination with 10 $\mu$ mol/L ER antagonist (ICI), 10 $\mu$ mol/L EGFR inhibitor (AG), 10 $\mu$ mol/L MEK kinase inhibitor (PD), 10 $\mu$ mol/L protein kinase C inhibitor (GFX), 10 $\mu$ mol/L protein kinase A inhibitor (H89), 10 $\mu$ mol/L phosphoinositide 3-kinase inhibitor (WM). ERK2 serves as a loading control. Data shown are representative of three independent experiments.

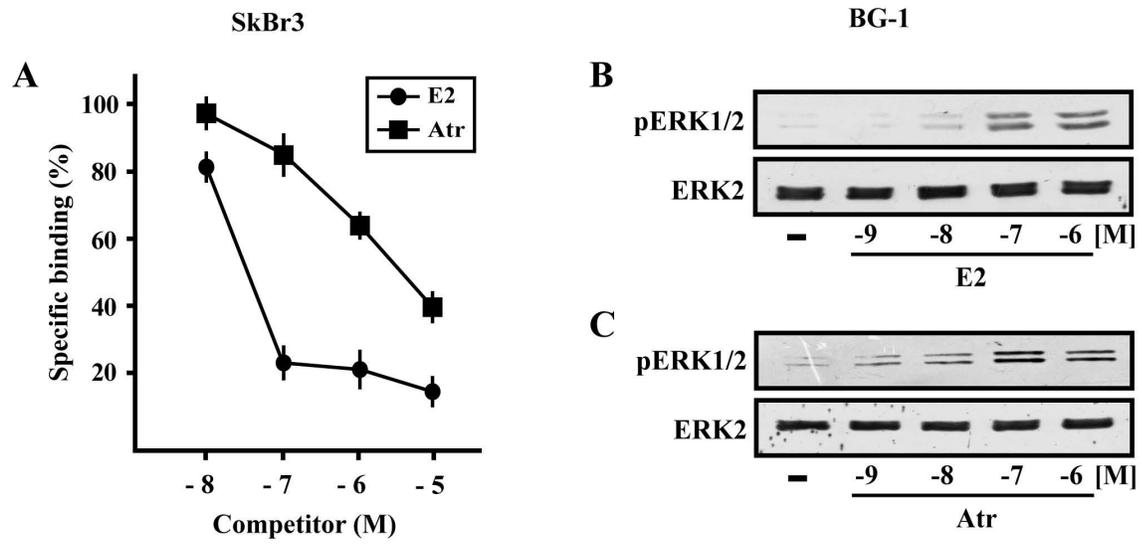
**Figure 4.** Proliferation of BG-1 and 2008 cells exposed to E2 or atrazine (Atr). Proliferation of BG-1 (A) and 2008 (E) cells in response to increasing concentrations of E2 or Atr. Proliferation of BG-1 (B–D) and 2008 (F–H) cells treated as indicated with vehicle (–), 100nmol/L E2 or

1 $\mu$ mol/L Atr, 10 $\mu$ mol/L AG, 10 $\mu$ mol/L PD (B, F) or transfected with control siRNA or siRNA ER $\alpha$  (C, G) or transfected with control shRNA or shGPER (D, H). Proliferation of cells receiving vehicle was set as 100% and the cell growth induced by treatments was calculated. Values shown are mean  $\pm$  SD of three independent experiments performed in triplicate. ( $\bullet$ ,  $\circ$ ,  $\blacksquare$ ,  $\square$ ,  $\ast$ ,  $\Delta$ ) indicate  $p < 0.05$  for cells receiving vehicle (–) versus treatments.

**Figure 5.** ERK1/2 phosphorylation (A) and c-fos and CTGF expression (B) in CAFs silenced for GPER expression and treated with vehicle (–), 1nmol/L E2 or 1 $\mu$ mol/L atrazine (Atr). The efficacy of GPER silencing was ascertained by immunoblots, as shown in side panels. ERK2 and  $\beta$ -actin serve as loading controls. Data shown are representative of three independent experiments. (C) The migration of CAFs induced by 100nmol/L E2 and 1 $\mu$ mol/L Atr is prevented knocking down GPER and CTGF expression. Migration of cells receiving vehicle was set as 100%, and the cell migration induced by treatments was calculated. Values shown are mean  $\pm$  SD of three independent experiments performed in triplicate. ( $\circ$ ) indicates  $p < 0.05$  for cells receiving vehicle (–) versus treatments.



**Fig.1**



**Fig.2**

BG-1

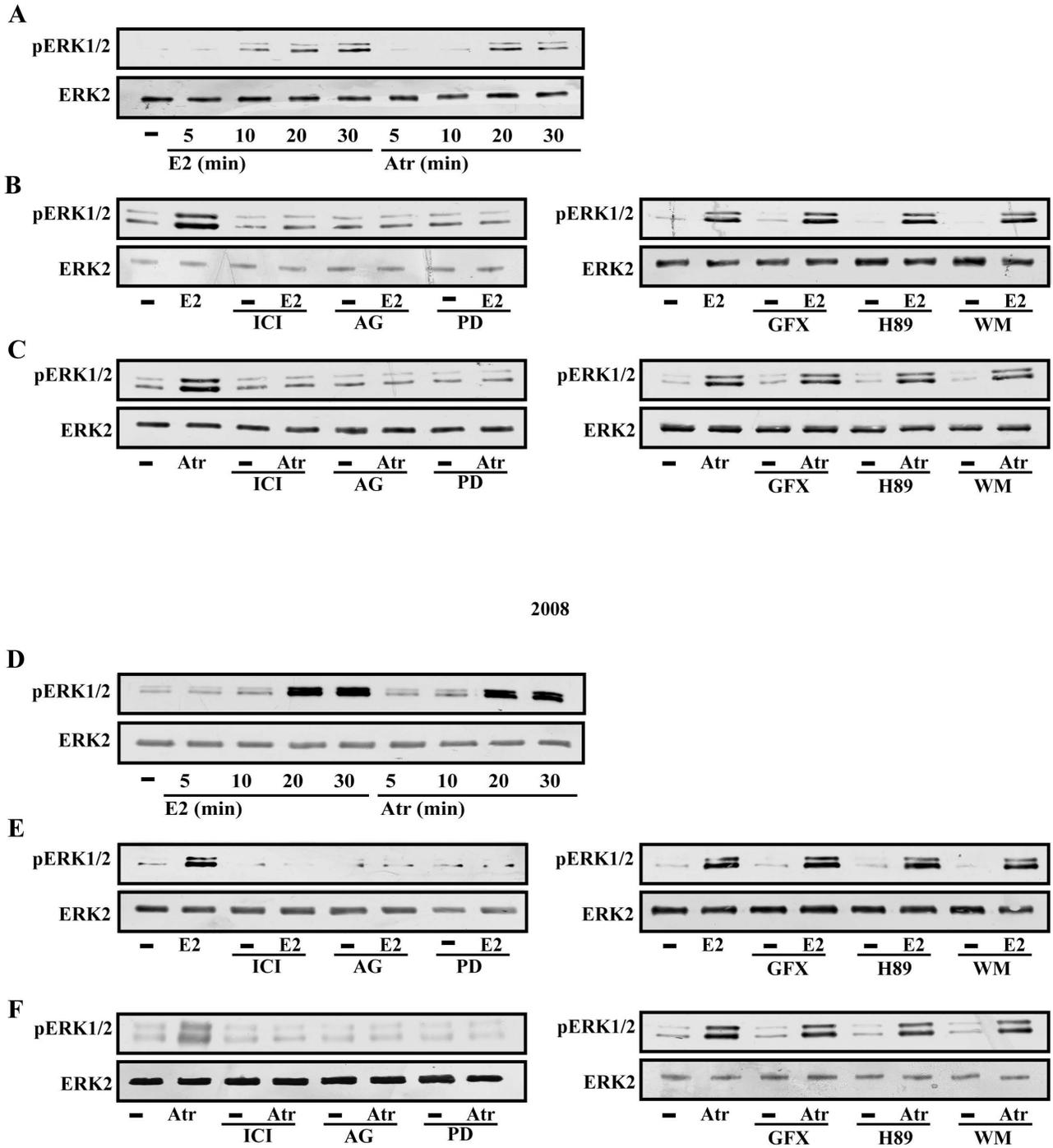
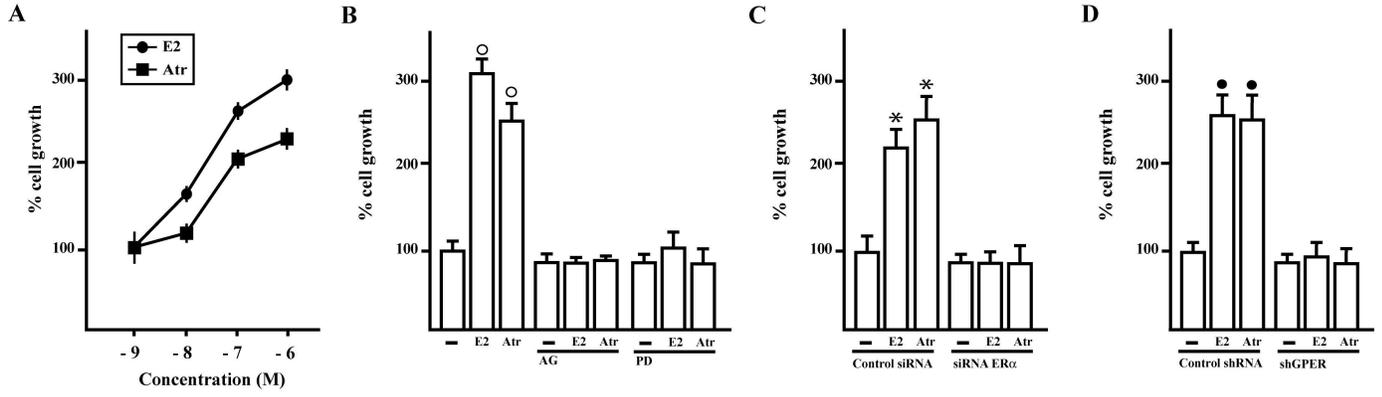


Fig.3

BG-1



2008

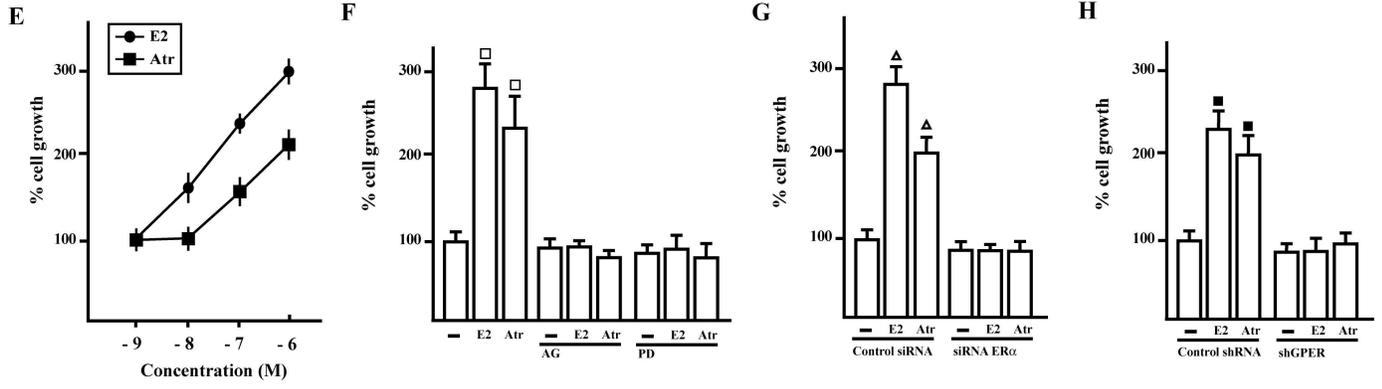
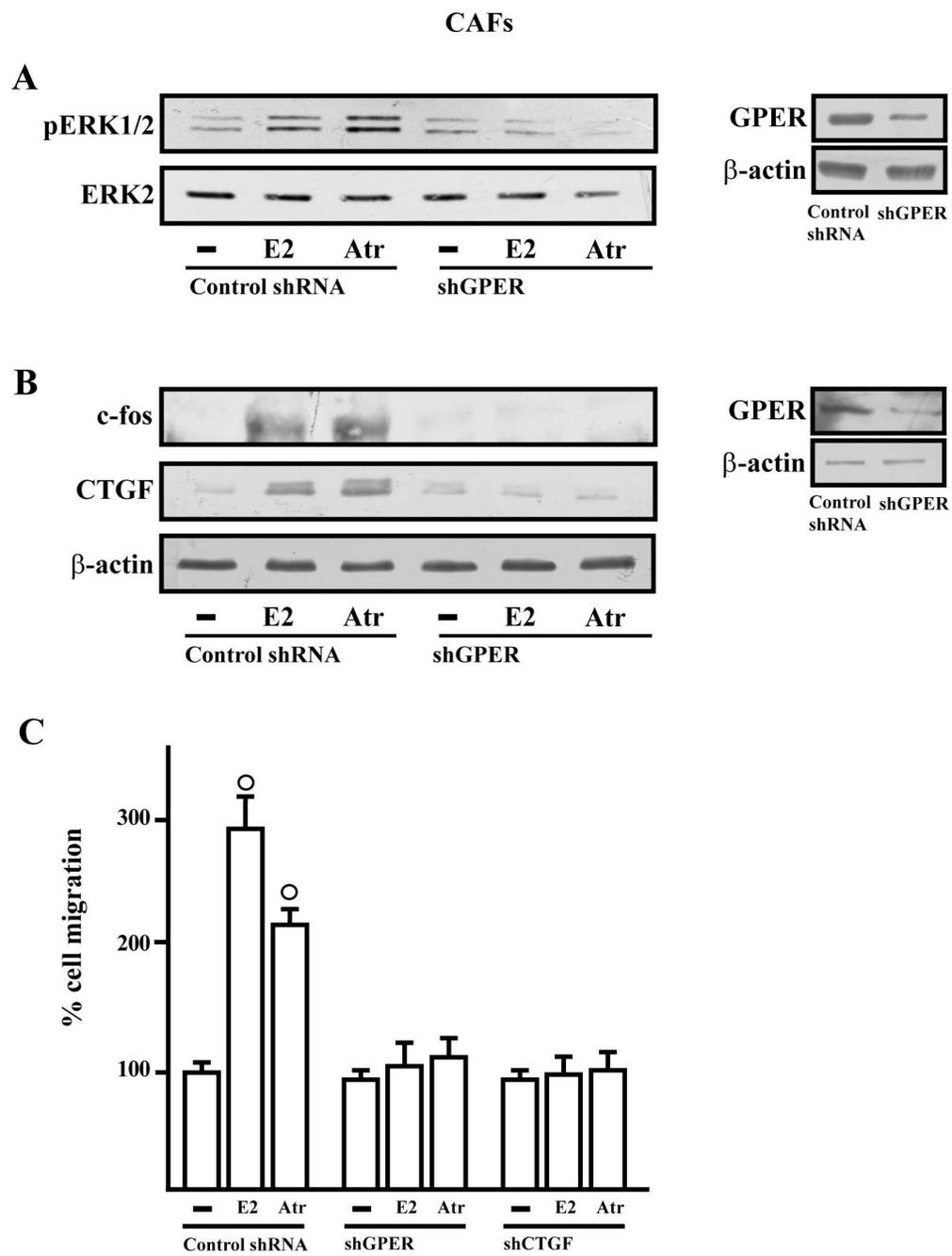


Fig.4



**Fig.5**